

Stem Cell Assays in the Evaluation of Myelotoxicity

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The concept and characteristics of different types of hematopoietic cells have been described. Hematopoietic stem cells are currently considered to exist in a variety of populations with different degrees of commitment towards a particular cell line. By a combination of animal studies and studies with hematopoietic disorders, the concept of dividing hematopoietic stem cells into uncommitted and committed types has emerged in the past several years. Uncommitted stem cells are capable of differentiation, under the proper stimulus, into either of the cell lines of the hematopoietic system. These cells form a resting population of cells with a low mitotic rate and a long resting (G_0) phase. The committed stem cells are partially differentiated and mature only into one type of cell. The committed stem cell population is relatively more active than the population of uncommitted stem cells.

A variety of assays both *in vivo* and *in vitro* are currently available for the study of different hematopoietic stem cells. These assays are semiquantitative. The number of colonies of mature cells which develop after the infusion or plating of a population of cells containing the stem cells is proportional to the total number of cells infused.

A variety of toxic as well as biological substances have been assayed in these systems and a quantitative depression of the number of colonies produced has been noted by a variety of workers. The degree of depression in the number of colonies varies with the agent in use and the type of assay employed. These studies have demonstrated that toxicity of chemicals on the hematopoietic stem cells can be studied with these *in vitro* and animal studies to give an assessment of their potential toxicity in the intact organism.

The recent development of the Dexter two-layer liquid culture system has provided a new impetus to the research on the uncommitted stem cell in a variety of organisms.

Most cell systems in mammals are comprised of differentiated cells with little or no self renewal. However, the gastrointestinal mucosa, skin, and the hematopoietic system have a large turnover and self renewal capability. This self renewal depends on a cell population referred to as stem cells. These cells are characterized by the dual capacity of self renewal and of differentiation into more mature types of cells. Stem cells have been studied in great detail in the hematopoietic system largely due to the easy accessibility of the cells (1).

Hematopoietic stem cells exist in different "populations" which are based on the degree of commitment to one or other cell type. Through a large amount of *in vivo* and *in vitro* work in humans and

animals, a number of biological features of these cells have been defined. It has been shown that there exists in the hematopoietic system a popula-

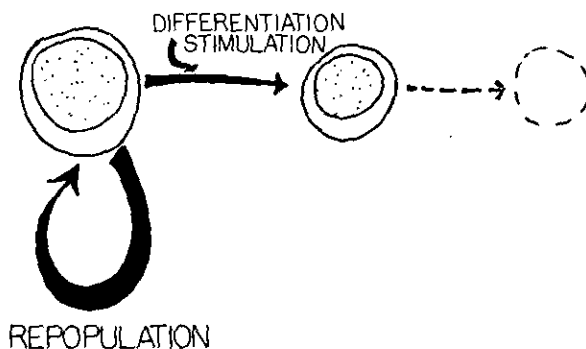


FIGURE 1. Schematic representation of the pathways followed by a stem cell. Note the need for a stimulus for differentiation and the high priority for self renewal.

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tion of uncommitted or pluripotent stem cells capable of giving rise to another population of stem cells committed to different cell lines. Committed stem cells for the granulocytic series, the erythroid series, lymphocytes, and megakaryocytes have been defined. In this paper, we will briefly review the current knowledge of the characteristics of the hematopoietic stem cells, the assays employed for their study, and the potential use of such assays for determining myelotoxicity.

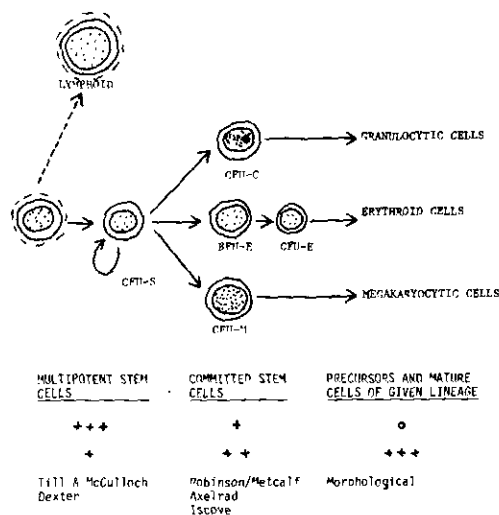


FIGURE 2. Schematic representation of the interrelationship of the different stem cell populations and the morphologically recognizable precursors of blood cells.

Characteristics of Stem Cells

At the present time, stem cells are recognized by their functional characteristics and have not been identified morphologically. The concept of the stem cell is that of a cell population which has the capability of differentiating, under appropriate stimuli, into more mature cells and at the same time have a potential for self renewal. The first feature provides the immediate needs in the peripheral blood and the self renewal capacity provides a reservoir of precursor cells for long-term needs. It may be mentioned at this point that the self-renewal capacity is less striking in the committed stem cells as compared to the uncommitted stem cells.

The factors controlling egress of pluripotent stem cells into the committed compartments are poorly understood. The stimulus for differentiation of committed stem cells is humoral in nature. The best characterized of the humoral factors is erythropoietin (2). A factor with activity on committed granulocyte stem cells has also been characterized and studied in detail. This is referred to as colony-

stimulating factor (CSF) or colony-stimulating activity (CSA) (3).

Studies of the pluripotent stem cell are based on *in vitro* and *in vivo* techniques which measure the differentiated progeny of the stem cell. The kinetics of these stem cells have been studied primarily by disturbing their equilibrium by inducing changes in the progeny. For example, the induction of anemia leads to the mobilization of stem cells towards increased production of red cells while polycythemia suppresses erythropoiesis. These phenomenon can be easily quantitated and studied. Tritiated thymidine suicide experiments have shown that most pluripotent stem cells are in the resting or G_0 phase. Thus, a large proportion of stem cells are normally not undergoing DNA synthesis. At the same time, it has been shown that after perturbation of stem cells, the DNA synthetic rate increases in proportion to the need for mature cells in the peripheral blood.

Types of Hematopoietic Stem Cells

Since the 1920's, based on the work of Maximow, the concept of hematopoietic stem cells as precursors for erythrocytes, different types of leukocytes, and platelets has been accepted. There has, however, been a considerable amount of debate as to whether there was a single pluripotent stem cell for all cell types or different types of unipotent stem cells for each cell line. More recent evidence has shown that there exists in the hematopoietic system (in all species studied) a pluripotent stem cell and also unipotent stem cells committed to differentiation towards each of the cell lines. These stem cells have been identified by the nature of their progeny and by the response to different types of stimuli. Kinetic differences between uncommitted pluripotent stem cells and the stem cells committed to erythropoiesis and granulopoiesis have also been shown.

Uncommitted Pluripotent Stem Cells

This is a resting population of cells which are capable of giving rise to cells of different lineages in the hematopoietic system. Granulocytic, erythroid, and megakaryocytic cell lines are derived from these cells. Lymphocytes and fibroblasts in the marrow may also be derived from an earlier form of the pluripotent stem cells.

This cell population has been identified by a number of techniques especially in mice. The Till and McCulloch technique (4) (described in detail

granulocytic, erythroid, megakaryocytic, and mixed colonies when syngeneic bone marrow cells are given intravenously to lethally irradiated mice. By manipulation of the status of the recipient or the donor mouse, it has been possible to show that this stem cell is responsive to humoral stimuli (5).

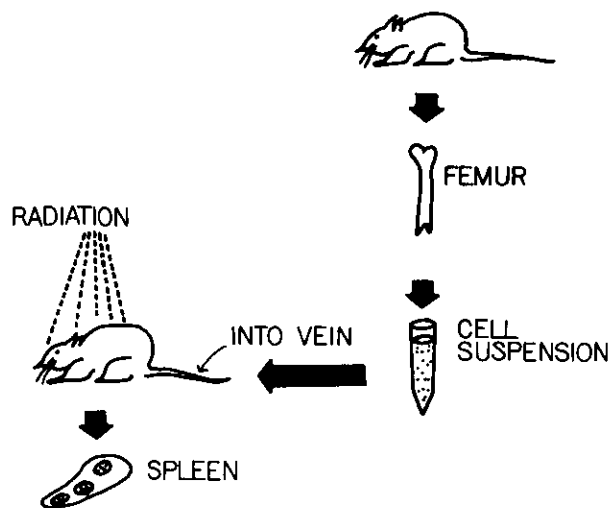


FIGURE 3. Outline of the Till and McCulloch assay for pluripotent stem cells in mice (CFU-S).

Evidence for a pluripotent stem cell in the human comes from studies of diseases like chronic myeloid leukemia and polycythemia vera. Clinical and hematological observations, chromosomal analysis (6), and analysis of glucose-6-phosphate dehydrogenase isomers (7) have shown a clonal abnormality in both these diseases. It has also been demonstrated that a clonal abnormality is present in the granulocytic, erythroid, and megakaryocytic cell lines. Fibroblasts and lymphocytes are free of this clonal abnormality.

The most definitive assay and method of demonstration of the pluripotent stem cell is the Till and McCulloch technique. Since this leads to colony formation in the spleen, this cell is commonly referred to as the colony forming unit in spleen (CFU-S) and is the best model for pluripotent stem cells. CFU-S have been demonstrated to be present in the bone marrow, spleen, fetal liver, and also circulating in peripheral blood. Further, the colonies formed in the recipient mice also contain CFU-S capable of forming secondary colonies in a second recipient.

Morphological identification of CFU-S is not possible at the present time. A number of cell separation techniques suggest that CFU-S fractionate with mononuclear cells with morphology

experiments have shown that CFU-S are capable of repopulating the bone marrow and of restoring normal hematopoiesis after it has been destroyed by radiation, drugs, or other mechanisms.

Assay of CFU-S

Spleen Colony. The most commonly employed assay for multipotential stem cells is the technique of Till and McCulloch (4). This assay was first described in 1961 and has become the most standard assay for the multipotential stem cell. In fact, the cell assayed in this system, CFU-S, is considered to be, for practical purposes, synonymous for the multipotential stem cell.

Syngeneic mice are employed in this assay. One set of mice is given lethal radiation, approximately 700 rad, to produce destruction of all endogenous hematopoietic tissue in the bone marrow and spleen. Bone marrow cells from unirradiated syngeneic mice are given intravenously. After 8-10 days, the recipient mice are sacrificed at which time macroscopic nodules of hematopoietic cells are seen in the spleen and the bone marrow. These consist of erythroid, granulocytic, megakaryocytic, and mixed colonies. It is the formation of these colonies in the spleen that gives the name colony forming unit-spleen for the cells assayed by this system.

The number of colonies formed is proportional to the number of bone marrow cells infused. This assay can be used to study, in a quantitative manner, the kinetics of CFU-S. At the same time, humoral and other influences on the CFU-S can be studied. For example, if the recipient mice are rendered anemic, a larger number of erythroid colonies are seen as compared to the other types. On the other hand, if the recipient mice are made polycythemic very few erythroid colonies are seen. Erythroid differentiation can be induced by exogenous erythropoietin. The self-renewal potential of the CFU-S can also be demonstrated in this assay. If colonies from the spleen are dissected, resuspended, and infused into a second set of irradiated syngeneic hosts, further colony formation is noted, demonstrating thereby the presence of multipotential stem cells in the colonies which showed differentiation into one or more cell lines. Not directly a part of the assay but of biological significance is the distribution of colonies in different parts of the bone marrow and spleen; erythroid colonies are more often seen on the surface while granulocytic and megakaryocytic colonies are usually deeper.

Endogenous CFU-S can also be demonstrated by modification of this technique. If mice are given lethal radiation with shielding of one leg, hema-

seen in the spleen after 10-15 days. These colonies have essentially the same characteristics as those obtained from syngeneic mouse bone marrow. These latter experiments demonstrate the capability of stem cells to circulate in the blood and reseed the bone marrow and spleen.

Dexter Culture. This is a relatively new technique (9) for culture of hematopoietic cells which as yet has not been fully exploited. However, it offers a great potential for study of hematopoietic stem cells, both the uncommitted and the committed.

In this system, bone marrow cells are grown in tissue culture flasks. The culture flasks are incubated in an atmosphere of 5% CO₂ in air at 37°C for 3 weeks. During this period, the medium and nonadherent cells are removed twice weekly and replaced with fresh medium. After approximately 3 weeks, an adherent layer is formed in the tissue culture flask. The adherent layer consists of phagocytic mononuclear cells, "epitheloid" cells, and giant fat containing cells. Once a monolayer is formed, a second batch of bone marrow cells is added to this culture. The environment provided by the adherent cell layer allows the proliferation of stem cells in the culture system. Both pluripotent stem cells (CFU-S) and granulocytic committed stem cells [colony-forming unit in culture (CFU-C)] proliferate in the nonadherent layer in the secondary culture. (CFU-C are described in further detail below.) The Dexter culture is a means of amplification of CFU-C and CFU-S and thereby provides an easily accessible source of these stem cell populations.

To date, the potentials of the Dexter culture have not been fully utilized but it can be seen that this culture system would allow the study of a large number of modifications for an understanding of the basic biology of stem cells and also a means of studying changes in them by drugs and other modifiers.

Use of CFU-S for Toxicity Studies

The CFU-S assay for stem cells has been used for study of drug/chemical toxicity by a number of workers. Fostad and Pihl (10) studied ricin and abrin, two toxic plant lectins with cancerostatic properties, in the CFU-S system. They also studied adriamycin and 5-fluorouracil, two standard cancer chemotherapeutic agents, in the same system. The drugs were given to donor mice in an escalating dose schedule. Three days later their bone marrow cells were infused into radiated recipients. The number of CFU-S obtained from the femurs of treated mice decreased progressively as the dose of

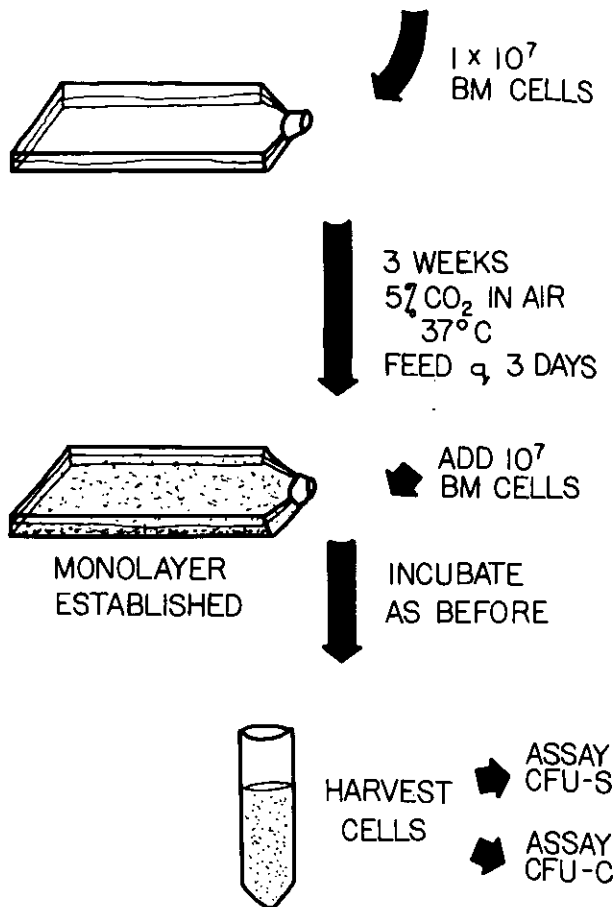


FIGURE 4. Outline of the Dexter assay for CFU-S and CFU-C *in vitro*.

drug increased over a three-fold range. The higher dose was near the LD₅₀.

Uyeki et al. (11) used CFU-S and CFU-C assays to study the effect of benzene inhalation on mice. They found bone marrows from mice exposed to benzene produced 7.5 ± 0.3 colonies/10⁵ cells as opposed to 18.7 ± 0.7 colonies in control animals. Thus, the pluripotent stem cell, as measured by the CFU-S, is responsive to the effect of known myelotoxic agents and a dose relationship is suggested by the available data. Data obtained by using the Dexter culture for study of toxins are not available at the present time.

Committed Unipotent Stem Cells

Granulocyte Committed Stem Cells

The granulocytes include neutrophils (polymorphonuclear leukocytes-PMN), eosinophils, basophils,

have been studied in some detail and a fairly clear picture of the later stages of their kinetics has emerged (12, 13). Granulocytes are derived from a committed stem cell commonly referred to as the colony forming unit in culture (CFU-C). The maturation of CFU-C depends on a stimulus commonly referred to as colony stimulating factor (CSF) (3). CSF is heterogeneous and, at the present time, its chemistry is poorly worked out. However, a number of subtypes of CSF have been recognized with activity of different types (3).

A morphologically identifiable population of dividing and maturing cells is derived from the CFU-C. This proliferates in the bone marrow and leads to amplification of the granulocyte precursors. From the mitotic compartment (myeloblasts, promyelocytes, and myelocytes), a pure maturation compartment of cells is derived (metamyelocytes and bands) which is present only in the bone marrow. Finally, mature granulocytes emerge from the bone marrow into the blood. The total time period for the maturation of myeloblast to neutrophil is approximately 10 days. There is a large reserve of mature granulocytes in the bone marrow.

In the peripheral blood, neutrophils are present in two interchangeable compartments, the circulating granulocyte pool (CGP) and the marginated granulocyte pool (MGP). The two together constitute the total blood granulocyte pool (TBGP) (13). The circulating granulocyte pool is sampled by the usual techniques of venipuncture while the marginated pool cannot be sampled by this technique. Demargination occurs under the influence of a number of factors, particularly the release of epinephrine.

The egress of granulocytes from peripheral blood into the tissues is exponential, and the half life of granulocytes in circulation is 6-8 hr. These data are of importance because the reserve of granulocytes in the bone marrow and the peripheral blood MGP may produce a discrepancy between the degree of granulopoiesis and the total number of granulocytes sampled from blood. This, combined with the time lag in the maturation of myeloblasts to granulocytes and the various factors responsible for the release of granulocytes from the bone marrow, makes the quantitation of granulopoiesis somewhat difficult. Assessment of granulopoiesis from changes in peripheral blood is not accurate and is better studied in the context of the changes in the early phase in the bone marrow rather than from the changes in peripheral blood.

CFU-C Assay. The CFU-C and its assay was first described in 1965 for mice (14, 15) and for

bone marrow cells are plated in semisolid agar with nutrients and the stimulus (CSF). The source of CSF can be from urine, peripheral blood cells (16), or from placental conditioned medium (3). After 7-14 days of incubation in an atmosphere of full humidification with 7.5% CO₂ in air at 37°C, discrete colonies of mature granulocytes are seen. These colonies have 50 to 500 cells and also smaller clusters of cells are seen under certain circumstances. These colonies consist of mature granulocytes and, in some cases, macrophages. It has been demonstrated that these colonies are clonal in nature (17). There is a semiquantitative relationship between the number of bone marrow cells plated and the amount of CSF provided to the number of colonies formed (12). By keeping a constant number of bone marrow cells in the system, CSF can be assayed, and by maintaining a constant amount of stimulus, CFU-C can be assayed. At the same time, if both CSF and CFU-C are kept constant, the addition of other stimuli or inhibitors can be studied and the changes produced can be measured.

Use of CFU-C for Toxicity Studies. Uyeki et al. (11) used the CFU-C assay to study the effects of benzene inhalation on hematopoiesis in mice. Mice were exposed to benzene vapors (4680 ppm) for 8 hr. The mice were sacrificed on days 1, 4, and 7. Bone marrow was taken from the exposed mice and plated with colony stimulating factor. The results were expressed as percentage of control using the number of colonies formed from 10⁵ bone marrow cells and also by the total number of CFU-C colonies from one femur. A significant decrease in CFU-C was noted from femurs obtained one day after benzene inhalation. On day 1, the value was approximately 39% of control. By the fourth day, this had increased to 66% and by day 7, it was up to 77%. This suggests the toxicity of benzene on CFU-C is reversible with time.

Schein et al. have studied the effect of a number of drugs on the CFU-C (18, 19). They have shown a dose related inhibition of CFU-C from the bone marrow of mice treated with these drugs.

Interferons are glycoproteins produced by a variety of vertebrate cells in response to viral infections. Apart from the antiviral effect, they have been shown to have a number of effects on cellular functions (20, 21). Hull et al. (22) studied the effect of human and mouse interferons on CFU-C and CFU-E. They noted a consistent inhibitory effect of interferon on growth of both types of committed stem cells in humans and in the mouse system. There was a dose-related response noted. Further, they noted a differential effect with

effect on CFU-C and erythroid committed stem cells, (CFU-E). Human fibroblast and human leukocyte interferon inhibited the growth of the CFU-E equally. However, with the CFU-C system, human leukocyte interferon was much more potent and produced a 50% inhibition of colony formation at a concentration approximately 1000-fold less than that required with human fibroblast interferon. These data point to the specificity of the action of interferon and, more importantly, point to the quantifiable nature of this assay for different types of inhibitory activity.

Nissen et al. (23) also reported the response of human CFU-C to interferon. They also found a dose related inhibition of CFU-C when cultures were grown in the presence of interferon.

In view of the recent interest in interferons, particularly as anticancer agents, it may be worthwhile to mention some aspects of the relationship of interferon to CSF. Both CSF and interferon are glycoproteins with similar electrophoretic ability. Further, the production of both is stimulated by synthetic polynucleotides, particularly Poly I-Poly C. The biological significance of these observations is not clear but raises the speculation that interferons and CSF both maybe physiologically important substances related to cell regulation.

Erythroid Committed Stem Cells

The kinetics of red blood cells is much simpler than the kinetics of granulocytes. Erythroblasts which develop from erythroid committed stem cells differentiate and mature in the bone marrow with a progressive decrease in total cell size and nuclear size. There is also a progressive increase in the hemoglobin content of the cells. By the stage of the reticulocytes, the nuclear material is extruded and the cell is completely hemoglobinized. The precursor cell, up to the stage of reticulocyte, matures entirely within the bone marrow. The reticulocytes spend approximately 24 hr in the bone marrow and subsequently circulate as reticulocytes in peripheral blood for another 24 hr. The reticulocytes then mature into adult red cells which have no nuclear material and are largely concerned with transport of oxygen through their hemoglobin content. Red cells spend approximately 120 days in circulation, at which time following a process of senescence, they are destroyed by the cells of the reticulo-endothelial system. Unlike granulocytes, red cells spend their entire life span within the vascular compartment and their loss is age dependent and, therefore, not exponential. In humans, at least, there is minimal amount of storage of red cells in

these reasons, the assessment of erythropoiesis on the basis of changes in the peripheral blood is relatively easy.

The erythroid committed stem cells have been studied in some details. *In vivo* and *in vitro* studies have shed a considerable amount of light on the nature of these stem cells. *In vivo*, it was first demonstrated in the 1950's that the production of polycythemia in radiated mice led to a lack of erythroid differentiation which could be reversed by the addition of erythropoietin (24). Thus, the concept of the erythropoietin responsive cells (ERC) was developed. The ERC were further studied *in vitro*. Studies of the kinetics of this cell and also its responsiveness to different stimuli have demonstrated the existence of at least two populations in ERC. These have been classified into the burst-forming unit-erythroid (BFU-E) and the colony-forming unit-erythroid (CFU-E). The burst-forming unit is considered to be the more primitive cell; it responds to higher concentrations of erythropoietin, takes a longer time in culture, and produces relatively larger colonies. The CFU-E, on the other hand, is derived from the BFU-E, it requires lower concentrations of erythropoietin, takes only 3-4 days of culture and produces small clusters of erythroid cells (25-27). More recent data have suggested that the activity of the erythropoietin containing preparations may have a separate stimulus which acts on more primitive cells and this activity has been referred to as burst promoting activity (BPA) (28).

Assay of Red Cell Precursors. Both liquid culture and semisolid culture systems have been employed for assay of red cell precursors. However, the liquid culture system mainly studies the hemoglobin synthesis by erythroblasts which do not have stem cell characteristics and are relatively late forms of erythroid precursors. This system will not be discussed in any detail though it has been described elsewhere (29).

The semisolid culture system has two variants, both of which provide the same type of information and are usable for the study of stem cells, the various stimuli which affect the stem cells, and also to study the effect of exogenous agents. Like the assay of CFU-C, these systems employ a source of stem cells (usually bone marrow), provide adequate amounts of nutrients, a method of stabilizing the cells and a source of stimulus. Incubation is carried out in an atmosphere of 5% CO₂ in air with full humidification at 37°C.

The plasma clot culture system first described by Axelrad and others has been used more commonly (25). However, the technique employing methyl-

methycellulose system has been described by Iscove and Sieber (26). Both these cultures are quantitative in their response to the various stimuli and also to the number of cells put into the culture. The Axelrad culture has been employed in a number of situations for study of toxic exogenous materials.

Use of CFU-E for Toxicity Studies. Hull et al. (22), as described earlier, employed CFU-C cultures to study the effect of interferon both in the human and the mouse system. In both systems, they demonstrated a quantitative relationship of the inhibition of colony formation by increasing concentrations of interferon. Approximately 50% reduction was noted at a concentration of 10^3 interferon units/ml of culture. The differential effect noted with leukocyte and fibroblast interferon on CFU-C was not seen with CFU-E.

Wallner et al. studied the effect of serum from patients with chronic renal failure on the growth of CFU-E. They found a quantitative relationship in the depression of colony formation by the addition of serum from patients with chronic renal failure but not from serum of normal controls (30).

Megakaryocyte Committed Stem Cells

At the present time, because of technical reasons, the feasibility of studies on megakaryocyte committed stem cells is limited. Even though megakaryocyte committed stem cells have been demonstrated by some workers, the growth of such colonies is possible under only very exacting conditions and their potential for use for the study of toxicity on the stem cell is relatively limited (31).

Conclusion

The hematopoietic stem cells provide a sensitive and relatively simple system for assessment of toxicity of potential environmental hazards. At different times in different assays, the effect of industrial chemicals, chemotherapeutic drugs, and biological materials has been demonstrated using assays of hematopoietic stem cells. The assays, by and large, have been demonstrated to be quantitative in nature and have a reasonable degree of specificity for the stem cells.

The conditions required for culture of hematopoietic stem cells are fairly exacting. However, at the same time, they allow a fair amount of flexibility in the amount of stimulus provided and the number of bone marrow cells put into a system. This flexibility should allow these techniques to be used for the study of a variety of toxic materials in these systems.

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